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# Characterization of Transgenic Mice Containing Adenovirus Early Region 3 Genomic DNA

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**Human adenoviruses (Ad) contain a complex transcription region (E3) which codes for proteins that interact with several arms of the immune system. However, E3 genes are not essential for replication in tissue culture. An E3-encoded 19,000-molecular-weight (19K) glycoprotein (gp19K) binds to the class I major histocompatibility complex (MHC) in the endoplasmic reticulum and prevents MHC transport to the cell surface. Three other E3 proteins are involved in the inhibition of apoptosis by tumor necrosis factor alpha. The entire E3 genomic DNA was utilized to produce transgenic mice to study the effect of the E3 proteins on pathogenesis of various infectious agents and to investigate the in vivo synthesis and processing of the multiple E3 mRNAs and proteins. There was basal expression of the E3 promoter in the thymus, kidneys, uterus, and testes and at all levels of the gastrointestinal tract. In addition, the E3 promoter of the transgene could be activated in some other organs, including the liver, by infection of these animals with an E3-deficient Ad (Ad7001) which contains a functional E1A region. Transactivation in vivo could also be demonstrated by infusion of bacterial lipopolysaccharide. There appeared to be differential ratios of expression between several of the E3 mRNAs in transgenic lung fibroblasts and primary kidney cells cultured from the transgenic animals. This observation suggested that there was differential mRNA splicing that was organ specific. These transgenic animals should provide a useful model for studying the effects of the E3 proteins on the immune system and on diseases affected either by control of MHC or by selected functions of tumor necrosis factor that are inhibitable by Ad E3 proteins.**

Adenoviruses (Ads) are important human pathogens which cause disease most commonly in the respiratory tract, conjunctiva, and intestine but also in the urinary tract and liver (27). Ads are being used as cloning vectors for the delivery of foreign genes into cells, and considerable expression of these nonviral genes has been obtained in tissue culture and in vivo (11, 22, 38, 42, 53). Thus, it would be helpful to understand more about Ad pathogenesis not only because Ads cause disease but also because they are potentially useful vectors for the delivery of genes for immunization as well as for gene therapy.

The mechanism of Ad pathogenesis is poorly understood. Although there are many viral mutants that affect various replicative processes in tissue culture, it has been difficult to distinguish the function of individual genes in the generation of disease. Human Ads do not replicate efficiently in rats or mice, but they have been studied in cotton rats, in which the virus does replicate (5, 6). It has been shown that deletions in early region 3 (E3) affect the pulmonary inflammatory response to Ad5 after intranasal inoculation of virus (15). The E3 region, which is not essential for the replication of Ads in tissue culture, codes for a number of proteins whose in vitro effects suggest that they are involved in pathogenesis. For example, the E3 19,000-molecular-weight (19K) glycoprotein (gp19K) binds to the class I major histocompatibility complex (MHC) heavy chain and retains the complex in the endoplasmic reticulum membrane. gp19K thus reduces the amount of class I MHC proteins on the cell surface and decreases CD8 lymphocyte recognition of the infected cell (6, 15). There are also three proteins that can decrease the cytolytic effects of tumor necrosis factor alpha (TNF-α), thus allowing infected cells to remain viable longer. The anti-TNF-α Ad proteins are the 14.7K protein, which acts alone (19, 21), and the 10.4K and 14.5K proteins, which function together as a complex (20). The 10.4K and 14.5K proteins can also accelerate the internalization of the epidermal growth factor receptor (47). In the cotton rat, deletion of gp19K from the infecting Ad has been shown to increase the pulmonary inflammatory response, and deletion of the 14.7K protein together with the 10.4K and 14.5K proteins converted a mononuclear pulmonary infiltrate into a polymorphonuclear cellular response (16, 17).

The Ad E3 transcription unit is a complex segment of DNA which contains at least nine open reading frames (ORFs) that could code for proteins greater than 6 kDa in size, and protein products from seven of these have been demonstrated (24, 52). (Fig. 1A). A variety of mRNAs originating from the single promoter are made by alternative splicing and terminate at either of the two potential poly(A) sites. One of the major protein products is the gp19K made from mRNA designated "a" and "c" in Fig. 1B (9). During viral infection, the Ad E3 promoter together with the other early Ad promoters requires transactivation by the Ad E1A for effective expression (4). This E3 activation has been studied also during transfection of the isolated promoter and can be achieved by both the human- and mouse-derived Ad E1A genes (3); however, the E1A protein does not bind directly to a DNA sequence in the promoter-enhancer but activates transcription factors which bind to the enhancer at their cognate sites (31, 33). Although E1A is generally necessary to activate Ad early promoters, it has been

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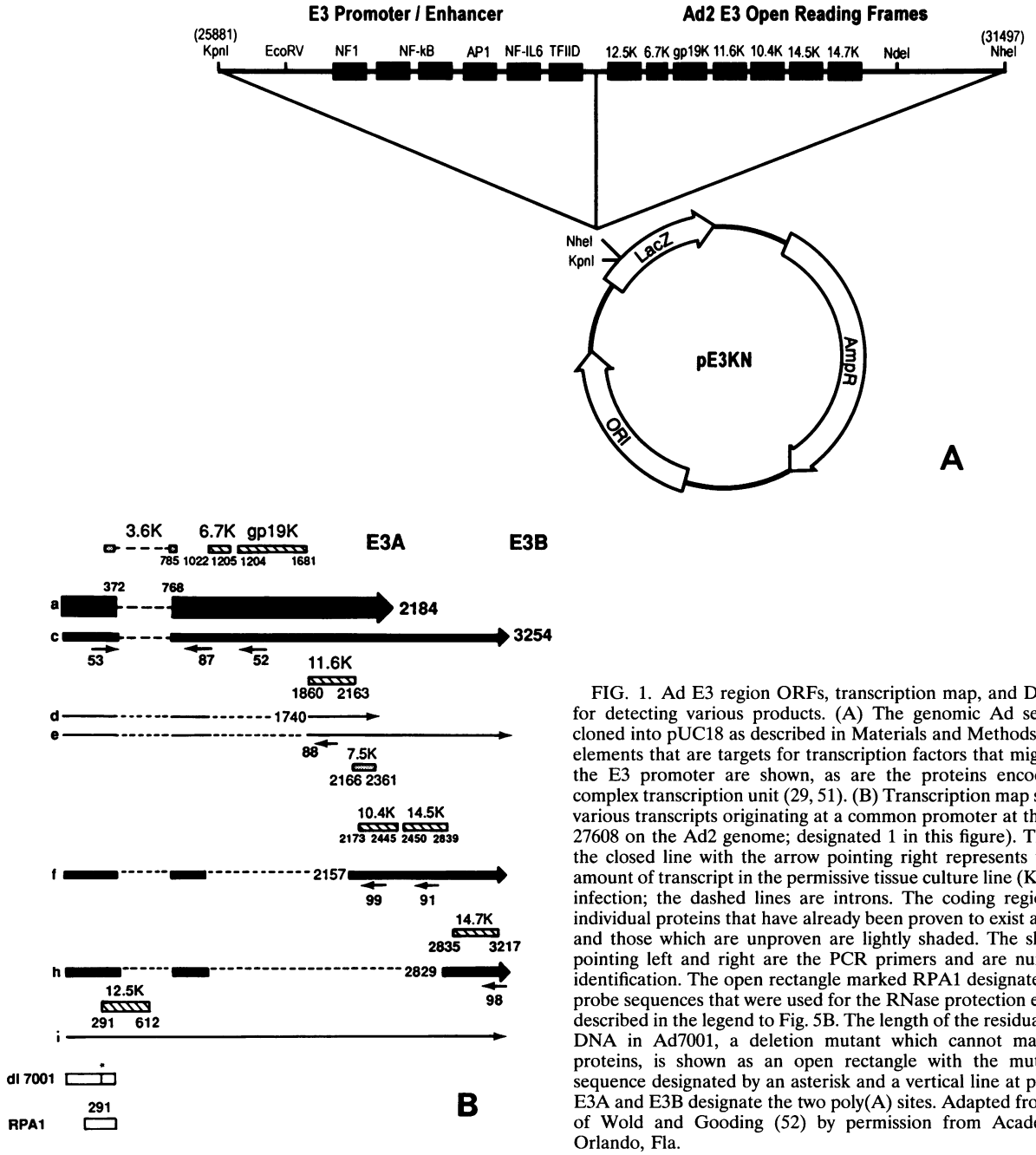



FIG. 1. Ad E3 region ORFs, transcription map, and DNA probes for detecting various products. (A) The genomic Ad segment was cloned into pUC18 as described in Materials and Methods. The DNA elements that are targets for transcription factors that might enhance the E3 promoter are shown, as are the proteins encoded in this complex transcription unit (29, 51). (B) Transcription map showing the various transcripts originating at a common promoter at the left (base 27608 on the Ad2 genome; designated 1 in this figure). The width of the closed line with the arrow pointing right represents the relative amount of transcript in the permissive tissue culture line (KB) after Ad infection; the dashed lines are introns. The coding regions for the individual proteins that have already been proven to exist are hatched, and those which are unproven are lightly shaded. The short arrows pointing left and right are the PCR primers and are numbered for identification. The open rectangle marked RPA1 designates the RNA probe sequences that were used for the RNase protection experiments described in the legend to Fig. 5B. The length of the residual E3 region DNA in Ad7001, a deletion mutant which cannot make any E3 proteins, is shown as an open rectangle with the mutated ATG sequence designated by an asterisk and a vertical line at position 291. E3A and E3B designate the two poly(A) sites. Adapted from the work of Wold and Gooding (52) by permission from Academic Press, Orlando, Fla.

shown that the Ad E2 promoter is active in F9 cells (28). Similarly, the E3 promoter was active without E1A in a lymphoid cell line, and the deletion of the NF-κB sites in the E3 promoter decreased this activity 30- to 40-fold, indicating the importance of NF-κB in promoter activity in lymphocytes (51). Other activators of the E3 promoter include TNF-α, which was shown to increase the amount of gp19K protein two- to threefold from plasmids transfected into 293 cells which also express Ad E1A (30). TNF-α could presumably activate E3 by inducing NF-κB1, NF-IL6, and AP1 (44), which have cognate recognition sequences near the E3 promoter. The cytokine interleukin-6 induces NF-IL6 in tissue cultures of hepatocytes, and the transactivation of the Ad E2 region which contains two NF-IL6 sites has been shown in these cells (45, 46). Presumably, the Ad E3 with a single NF-IL6 site would also be transactivated, but this has not been directly tested. All of these studies of the E3 promoter and its protein products have been done with tissue cultures. It would be important to know whether these effects occur in vivo, where they could alter the virus-host interactions and manifestations of Ad disease.

It would be important to further study the Ad E3 genes in vivo in an animal model for which the MHC has been well defined and for which there are good TNF reagents available. There are two murine Ads that have been isolated from mice and cause acute disease, particularly in young animals. The Ad FL, or mouse Ad type 1 (MAV-1), infects a variety of organs,

and MAV-2 infects the gastrointestinal tract (35). However, MAV-1 has been partially sequenced and found not to have an E3 region similar to those of most of the human Ads (40), and our attempts to place the human Ad E3 region into the truncated MAV-1 "E3" region have not been successful.

We have utilized two approaches to study the E3 genes in vivo. In one of them, we have cloned individual E3 genes into vaccinia virus (VV) and measured changes in VV pathogenesis in vivo (48). Using the isolated Ad 14.7K protein gene cloned into VV in the presence of the TNF-α gene also inserted into the virus (VV TNF+/14.7K+), we have shown that the virulence of VV after intranasal inoculation is increased by the Ad 14.7K protein. The increase in mortality, pulmonary inflammatory response, and the amount of virus that grew in the lungs of mice infected intranasally with VV TNF+/14.7K+ in comparison with the results in mice infected with VV TNF+/14.7K− (14.7K protein gene inserted in a nonexpressing orientation) clearly indicated that the in vivo mechanism of action of the 14.7K protein was to antagonize the effects of TNF-α. Although useful information can be obtained about the function of individual Ad genes in VV, other approaches are needed to understand the scope and complexities of the E3 effects on Ad infection and disease in vivo.

In a second approach, which is the subject of this paper, we produced transgenic mice carrying the Ad2 E3 region behind the native E3 promoter. This paper describes the generation of the Ad E3 transgenic animals, the expression of the various E3 mRNAs from this complex transcription unit, and the activation of the E3 promoter in vivo. These E3 transgenic animals should allow us to study the effects of the Ad proteins on the pathogenesis of disease as well as define some of the normal controls of gene expression that influence the amounts of these important proteins.

Transgenic mice have been used to study the pathogenesis of other infectious agents. The poliovirus receptor from human cells was inserted into mice to permit the use of these animals to differentiate between neurovirulent and attenuated strains of this virus (37). In addition, these animals have been used to show that poliovirus spreads to the central nervous system by retrograde transport along neural pathways (41). The human immunodeficiency virus type 1 *tat* gene was inserted into transgenic animals and induced dermal lesions resembling Kaposi's sarcoma and malignancies in the liver (49, 50). Hepatitis B virus transgenic animals have been made, and the effects of various cytokines, including TNF-α, on the down-regulation of the amount of hepatitis B virus mRNA have been determined. These transgenic approaches to study the pathogenesis of human viruses have been reviewed elsewhere (7, 32).

## MATERIALS AND METHODS

**Construction of Ad2 E3-containing transgenic mice.** An Ad2 DNA fragment (*Kpn*I at base 25881 to *Nhe*I at base 31497), containing the entire E3 region of the genome, was excised from viral DNA and cloned into the *Kpn*I-*Xba*I sites of pUC18, yielding plasmid pE3KN. The E3-region DNA for construction of the transgenic mice was released from the plasmid with the enzymes *Eco*RV and *Nde*I (Fig. 1A), separated on agarose by electrophoresis, eluted from the gel fragment, and utilized for the construction of transgenic mice according to standard techniques (25).

The mouse strain utilized for generation of the oocytes was Dba/2J ($H\text{-}2^d$), and the paternal component was B6/D2 F1 ($H\text{-}2^b$ and $H\text{-}2^d$); all backcrosses of the resulting transgenic animals were into DBA/2J. The animals were generated in the Transgenic and Stem Cell Facility at the Albert Einstein College of Medicine and have been maintained in a pathogen-free controlled environment. The initial assay to determine which progeny contained the transgenic DNA was by PCR on DNA obtained from the tails of the animals, and the genotype of each of the founders was confirmed by Southern blot hybridization.

**Isolation of DNA for PCR and Southern blot hybridization.** To detect the transgene with PCR, small tail pieces were digested in a buffer containing 10 mM Tris (pH 8.4), 50 mM KCl, 0.45% Nonidet P-40, 0.45% Tween 20, and 200 μg of proteinase K per ml overnight at 55°C. Proteinase K was then heat inactivated at 95°C for 15 min, and 5 μl of the samples was directly assayed by PCR. For Southern blots, a second sample of tail DNA was obtained and purified by digestion of the sample in 50 mM Tris (pH 8)–100 mM EDTA–0.5% sodium dodecyl sulfate–200 μg of proteinase K per ml for 16 h at 55°C, followed by three extractions with phenol-chloroform-isoamyl alcohol and ethanol precipitation. RNA was removed from the solubilized samples by RNase treatment, after which samples were extracted with phenol-chloroform-isoamyl alcohol and precipitated again with ethanol. Twenty micrograms of this DNA was cut with *Eco*RI, electrophoresed on 1% agarose gels, and transferred to Hybond N membranes (Amersham) by capillary blotting. The *Eco*RI D fragment of Ad2 DNA was labeled by random priming using [$^{32}$P]dCTP and used for blot hybridization at 42°C in the presence of 50% formamide overnight. The blots were washed under conditions for high stringency according to standard protocols (2).

**Preparation of RNA.** Total cellular RNA was purified by a modified guanidine isothiocyanate method (8) using the TRI reagent from MRC. Individual organs were homogenized in TRI with a Polytron homogenizer which was carefully cleaned between each sample; tissue culture cells were lysed directly in the culture vessel with TRI. RNA from these sources was prepared according to the instructions of the manufacturer. This RNA, which was essentially free of DNA, was used directly for RNase protection assays. For reverse transcription (RT)-PCR experiments, the RNA was digested with RNase-free DNase to remove any contaminating genomic DNA and the remaining RNA was extracted with phenol-chloroform-isoamyl alcohol and ethanol precipitated.

**RT.** Three micrograms of total cellular RNA was reverse transcribed with the Superscript system (Gibco/BRL) using oligo(dT) priming. The cDNA product was diluted in 1× RT buffer as indicated for each experiment and used for PCR.

**PCR.** PCR was performed in a 50-μl reaction mixture in which various dilutions of cDNA in a 3-μl volume had been added. The reaction mixture contained primer pairs (each was 100 ng in 1 μl), 4 U of *Taq* polymerase, 200 μM (each) deoxynucleoside triphosphates, 1.5 mM $MgCl_2$ in buffer, 50 mM KCl, 10 mM Tris (pH 9.0), and 0.1% Triton X-100. The sample was treated initially for 9 min at 94°C and then through 35 to 46 cycles (designated with each experiment) of 1 min at 94°C, 1 min at 60°C, and 1.5 min at 72°C before a final 10-min treatment at 72°C. Twenty microliters of the reaction mixture was analyzed on 2% Metaphor (FMC) or 1.2% regular agarose gels.

Sequences of primers used for PCR were as follows: GAT GACGATATCGCTGCGCTGGTCGTC and GAGCCTCAG GGCATCGGAACCGCTCG for β-actin; CTACGACTGAA TGACCAGTGGAGAG for primer 53 (Fig. 1B); GTCTGGT TACGCTCAGGCTGTAGG for primer 87; GGTCTGGCGC GTCTGCGCTTTAGG for primer 88; GAGTGCAGAGGTC CTCTGGACCCTG for primer 98; and GAAGGCGATGAC TACAGTGACGAG for primer 99.

**Quantitative PCR.** E3 mRNAs were quantitated by compet-

itive PCR essentially as described previously (14). After RT, constant amounts of cDNA products were coamplified with a series of dilutions of a competitor genomic DNA product of known concentration and a predicted size different from that of the cDNA product. Since the same primer pair amplifies both DNAs, the ratio of the amplified products was only a function of the target-competitor ratio of the original sample. Other variables, such as cycle number, primer dimer formation, or other individual sample variations, should not affect the results. The initial cDNA concentration can be calculated by determining the competitor concentration that gives the same intensity for target and competitor DNAs. To quantify overall transgene expression with this method, primers 53 and 87 were used to coamplify transgenic cDNA with linearized pE3KN as the genomic competitor by using various dilutions of cDNA and the number of cycles as indicated for each experiment. Initially, competitive PCR was done with 10-fold dilutions of the competitor DNA, and then the point of equivalence was determined more exactly by twofold dilutions. Since differences in the sizes of the target and competitor DNAs might influence the ratio of the products, the absolute amount of the original RNA might not be determined exactly. However, this technique should be very accurate in comparing relative levels of mRNAs in different samples.

**RNase protection assays.** To detect E3 mRNAs, a 150-bp Ad2 genomic fragment (RPA1; Fig. 1B) spanning a common part of all E3 transcripts was selected and amplified by PCR to detect E3 mRNAs. RPA1 was cloned between SP6 and T7 promoters by using the Bethesda Research Laboratories pAMP system. Primers used for this PCR were GTGCCT TCGGTTTACTTCAACCCC and GAGGTGTGTCAGGCG CAGTCGC. The cloned fragment was sequenced to determine the fidelity of copying. RNA probes were synthesized by using T7 polymerase and [$^{32}$P]UTP. Total cellular RNA was assayed with an excess of gel-purified RNA probe by using AMBION's RPA II kit according to the instruction of the manufacturer. For the detection of mouse β-actin mRNA, the pTRI-β-actin-mouse vector from AMBION was used. The protected RPA1 and β-actin fragments were separated on 6% polyacrylamide–urea gels and visualized either in a Phosphorimager (Molecular Dynamics) or by autoradiography on Kodak X-Omat film.

**Viruses and tissue cultures.** Wild-type Ad2 or Ad7001, which contains the E3 promoter sequences but does not code for any protein products from this region, was grown in HeLa suspension cultures and purified by two rounds of equilibrium centrifugation in CsCl gradients as previously described (26). Ad7001 is derived from the rec700 virus by deletion of most of the E3 from positions 362 to 3381. The truncated part of E3 remaining is homologous to the corresponding sequences in Ad2, except for an 8-bp mismatch at ATG 291 which was introduced when the virus was constructed (Fig. 1B) (39).

Primary kidney and lung fibroblast cultures were made from 6-week-old mice by the cold trypsin method essentially as described elsewhere (13) and maintained in 10% fetal bovine serum in Dulbecco's modified Eagle's medium. NIH 3T3 cells were maintained in 10% fetal bovine serum–Dulbecco's modified Eagle's medium.

Ad infections of tissue culture were with CsCl-purified viruses in monolayer flasks at a multiplicity of $3 \times 10^3$ virions per cell. Infections of animals were with $3 \times 10^{11}$ virions in 100 μl which was dialyzed free of CsCl for 6 h in Dulbecco's modified Eagle's medium with 10% glycerol.

**Immunoprecipitation.** Primary mouse kidney and lung fibroblast cultures ($10^7$ cells) were infected at a multiplicity of 50 PFU per cell for 16 h in the presence of 10 μg of cytosine arabinoside per ml. Proteins were labeled for 6.5 h with [$^{35}$S]methionine (200 μCi/ml), and the cells were harvested with Nonidet P-40 lysis buffer (150 mM NaCl, 1.0% Nonidet P-40, 50 mM Tris, pH 8.0) containing the protease inhibitors 2 μg of phenylmethylsulfonyl fluoride per ml, 100 μg of leupeptin per ml, 2 μg of pepstatin per ml, and 1 μg of aprotinin per ml. The Ad E3 gp19 protein was immunoprecipitated from the total lysate with a polyclonal rabbit antibody to gp19 and protein A-Sepharose according to standard protocols (23). Immunoprecipitates were separated on 10% polyacrylamide gels and visualized in the Phosphorimager.

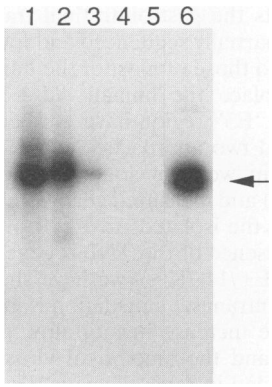
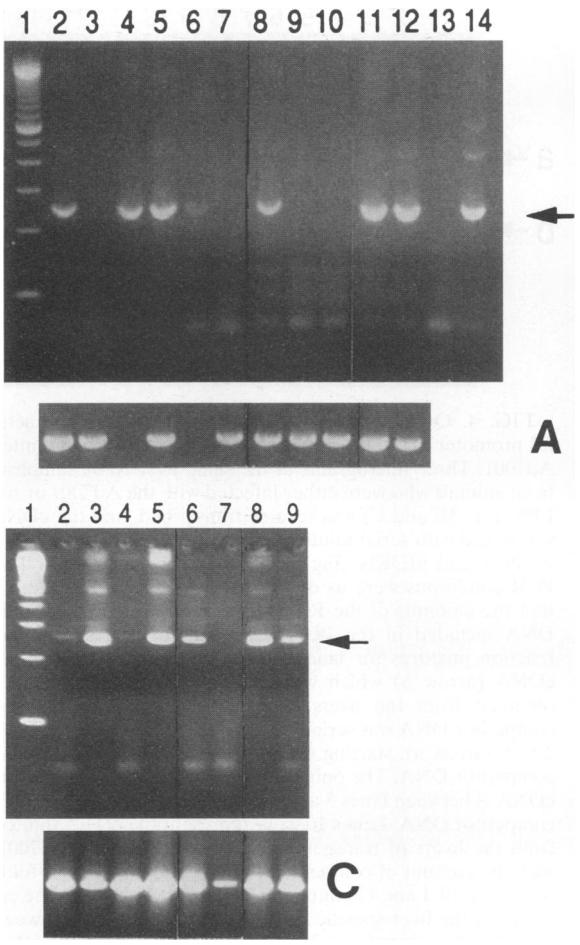



FIG. 2. Copy number of Ad E3 region DNA incorporated in transgenic founder animals. Genomic DNA was prepared from the tails of five putative transgenic animals and digested with *Eco*RI, which cuts twice within the inserted Ad sequence. The samples were electrophoresed in 1% agarose and analyzed for Ad sequences with the *Eco*RI D fragment probe which was labeled by random priming using [$^{32}$P]dCTP (with the Amersham Multiprime system). Lane 6 is the positive control, consisting of 30 pg of the Ad2 *Eco*RI D fragment. Each of lanes 1 to 5 contained 20 μg of mouse tail DNA from different potentially transgenic mice. Lane 2 represents the DNA from founder animal 8, which was the lineage studied in all the experiments described in this paper. The size of the expected fragment designated with an arrow corresponds to the single radioactive band observed.

## RESULTS

**Construction of transgenic mice and determination of copy number of inserted DNA.** Twenty-seven pups were born after the microinjection of the E3 DNA described in Materials and Methods. The pE3KN plasmid containing the entire E3 genomic region with its native promoter was prepared as described in Materials and Methods and is shown in Fig. 1A. The ORFs for the various proteins are shown, as are the transcriptional activation motifs contained in the promoter. Each E3 protein is synthesized from a single exon with its own translational start site and from differentially spliced mRNAs as shown in Fig. 1B (10). Since *Eco*RI cuts the transgene twice, it was anticipated that the same size of hybridizing band would be present in the DNA from each founder animal. After determination of which animals carried the transgene by PCR of tail DNA prepared as described in Materials and Methods, the DNA from positive animals was evaluated by Southern blotting to confirm the PCR results and to quantify the transgene copy number incorporated into the genome. The DNA from three transgenic animals is shown in Fig. 2 together with appropriate positive and negative controls. Copy number was calculated by comparing the intensities of the transgene bands in lanes 1, 2, and 3 to that of 30 pg of the control Ad2 *Eco*RI D fragment in lane 6. Two transgenic founder animals (lanes 1 and 2) had approximately 50 to 70 copies of the



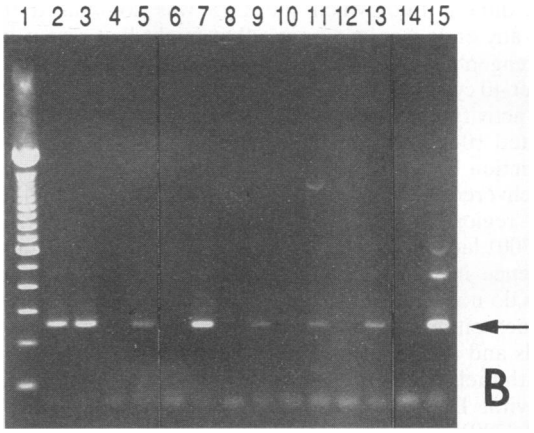




FIG. 3. Basal and induced expression of the Ad E3 promoter. (A) RNA from each of the designated organs was isolated, purified, and reverse transcribed from a transgenic mouse without any attempt to induce the promoter. PCR was performed for 40 cycles on 3 μl of undiluted sample from the RT reaction using primers 53 and 87 as described in Materials and Methods, and the specific band anticipated was 249 bp (arrow). Lane 1, molecular weight markers (100-bp ladders); lane 2, thymus; lane 3, spleen; lane 4, stomach; lane 5, small intestine; lane 6, large intestine; lane 7, liver; lane 8, kidney; lane 9, brain; lane 10, lung; lane 11, ovary; lane 12, testis; lane 13, negative PCR control; lane 14, positive PCR control using NIH 3T3 cells infected with Ad2 as a source of E3 region RNA (Fig. 1A). The β-actin control is shown in the insert beneath each of the lanes. (B) RNA from selected organs from a transgenic mouse, which had been stimulated by i.p. injection with 400 μg of LPS 16 h before sacrifice, was processed as for panel A. However, the DNA product of the RT reaction was diluted 10-fold, and PCR was performed for 38 cycles. As a control, the RNA from unstimulated animals was identically processed. Paired PCR results for each organ (unstimulated in even-numbered lanes; stimulated in odd-numbered lanes) are shown. Lane 1, molecular weight markers; lanes 2 and 3, thymus; lanes 4 and 5, spleen; lanes 6 and 7, liver; lanes 8 and 9, kidney; lanes 10 and 11, brain; lanes 12 and 13, lung; lanes 14 and 15, negative and positive PCR controls, respectively, as described for panel A. (C) RNA from an Ad7001-infected transgenic mouse or from transgenic tissue cultured cells which had been infected for 48 h was processed as for panel A. PCR was performed for 40 cycles using the undiluted cDNA samples generated by RT. Lane 1, molecular weight markers; lanes 2 to 5, from transgenic early-passage tissue culture cells (lane 2, uninfected kidney; lane 3, infected kidney; lane 4, uninfected lung; lane 5, infected lung). The rest of the lanes are from organs of infected animals: lane 6, spleen; lane 7, lung; lane 8, liver; lane 9, small intestine. The β-actin results are shown at the bottom of the panel.

transgene incorporated into their genomes, and the third animal (lane 3) appeared to have a single or a few copies of foreign DNA inserted. Lanes 4 and 5 are from animals that did not have detectable Ad DNA and were therefore negative littermates.

**Expression of the AdE3 transgene (basal and induced) in various organs of the transgenic animals.** In order to determine transgene expression in various organs, we initially screened the RNA samples by RT-PCR; subsequently, selected RNA samples were analyzed by quantitative PCR and/or RNase protection assays.

**(i) Basal activity.** For determining whether the E3 promoter was active in the various organs, RT-PCR (see Materials and Methods) was performed on the RNA purified from each of the organs from one animal of the multiple-copy lineage (Fig. 3A). The primers used for determining promoter activity were primers 53 and 87 (Fig. 1B), which spanned the first exon, a region contained in common in all of the previously mapped mRNAs. Since the sizes of the predicted PCR genomic and cDNA products would be different, this approach allowed the discrimination of the PCR products of the cDNA (249 bases) from those arising from any residual contaminating genomic DNA (643 bases). In animals that were not stimulated with any exogenous reagent known to induce the E3 promoter, specific Ad mRNA was easily detected in the thymus, stomach, small and large intestines, kidneys, ovaries, and testes (Fig. 3A). The β-actin PCR results are shown beneath each lane as a control for the integrity of the nucleic acid and the RT-PCR. The RT-PCR results were still positive for the stomach and large intestine, even though the β-actin signal seemed to be much weaker in these two organs. A similar pattern of expression was obtained from the other two lineages (data not shown).

**(ii) Induction by bacterial lipopolysaccharide (LPS).** Because the Ad E3 promoter has been shown to be transactivated by a number of different inducing factors in vitro, we utilized an approach that was anticipated to activate the promoter in additional organs in vivo. It has been shown that TNF-α activated the E3 promoter in plasmid transfection experiments and that TNF-α can be induced by LPS (1, 30). After the injection of LPS (*Escherichia coli* serotype O55:B5; Sigma) either intravenously (20 μg) or intraperitoneally (i.p.) (400 μg), inducibility of the E3 promoter as measured by RT-PCR was found in additional organs, such as the liver, lungs, spleen, and brain (Fig. 3B). The control mice received the same

amount of diluent into which the LPS was solubilized to control for any endogenous pyrogen that might be present in any of the reagents. Although there was basal expression in the kidneys after 40 cycles of PCR using undiluted cDNA (Fig. 1A, lane 8), no activity was detected after 38 cycles using the same cDNA diluted 10-fold (Fig. 3B, lane 8).

**(iii) Induction by Ad E1A.** The Ad E1A for transactivation could be delivered in the form of a virus that was lacking the normal E3 region. There are several of these viruses. The human Ad7001 lacks all the E3 ORFs, although it does contain some sequence including the promoter (Fig. 1B). Although human Ads do not replicate to produce much progeny virus in mouse tissues either in vivo or in vitro, they do attach to murine cells and produce early viral mRNAs, including E1A, together with their protein products. However, they synthesize very little viral DNA or late structural proteins. After i.p. injection, Ad7001 is found primarily in the spleen but also in the liver; other organs, such as the lungs, received very little virus in such a protocol (34). Another alternative is the mouse Ad (MAV-1) that also is deficient in E3 sequences and ORFs homologous to the wild-type human Ads but does replicate in some murine organs. Data from i.p. infection of a 7-week-old transgenic animal infected with $3 \times 10^{11}$ particles of Ad7001 for 16 h are shown in Fig. 3C (lanes 6 to 9) and demonstrate that the E3 promoter in the spleen and liver but not in the lungs was activated in vivo. This distribution is consistent with the previously demonstrated distribution of Ads after i.p. infection of mice (34).

Primary kidney and lung cultures from the transgenic animals have also been infected with the Ad7001 virus to study the E3 transactivation after direct infection of the target cells. Data from these experiments are shown in Fig. 3C (lanes 2 to 5). Although the E3 promoter was not activated in the lungs by i.p. infection with Ad7001 in vivo, the virus was able to activate the same promoter in short-term tissue culture of lung fibroblasts (Fig. 3C, lanes 4 and 5). Similar to lung fibroblast cultures, there was transactivation of the E3 promoter in kidney cultures infected with Ad7001.

**Quantitative analysis of transgene expression.** Quantitative RT-PCR experiments to compare the basal as well as the induced levels of transgene expression in different organs and primary cultures have also been done using some of the samples already known to be positive from the previous experiments. The PCR on cDNA was carried out in the presence of known amounts of competitor genomic DNA whose size was easily distinguished after electrophoresis of the products (see Materials and Methods). The genomic DNA contains the first intron, which is absent from all the cDNAs. With the common primers (primer 53 and 87) and various ratios of the known genomic DNA to the unknown amounts of RT cDNA product, it was possible to determine the amounts of the latter product of reverse-transcribed mRNA originally present in the samples. An example of this technique is shown in Fig. 4; the calculations for these and other organs tested for basal expression or after induction with LPS or infection with Ad7001 are summarized in Table 1. Comparative data for the liver stimulated by infection of transgenic animals with Ad7001 or infused with LPS are shown in Fig. 4. The dilutions of the competitor genomic DNA at which the intensities of the genomic and cDNA bands were similar indicated that the amount of E3 mRNA in the liver of a mouse treated with Ad7001 was approximately 1,000 times the amount in the transgenic littermate treated with LPS (Fig. 4). However, when twofold dilutions were done to obtain more-accurate numbers, the actual difference between Ad7001 and LPS enhancement in the liver was 310 (Table 1). Comparative data for the transgenic lung fibroblast cultures and kidney cultures infected with the Ad7001, using twofold dilutions of the competitor cDNA, are summarized in Table 1. Expression seemed to be the strongest in vivo after Ad7001 induction in the liver and spleen or in vitro in kidney and lung cultures. There also appeared to be significant basal expression in the small intestine, testes, and thymus. Although LPS induction of the transgene in the liver and spleen is readily detectable, it was quantitatively much weaker than induction with Ad7001.

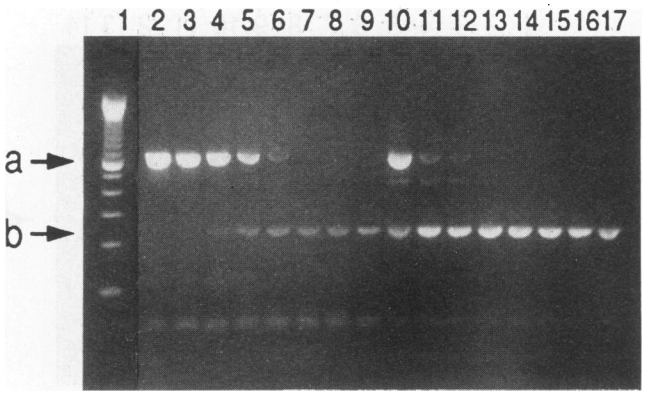



FIG. 4. Quantitation of RT-PCR. Comparison of the activity of the E3 promoter in the liver after stimulation with LPS and infection with Ad7001. Three micrograms of the same liver RNA samples extracted from animals who were either infected with the Ad7001 or treated with LPS (Fig. 3B and C) was reverse transcribed, and the cDNA product was mixed with serial dilutions of the Ad E3 genomic DNA contained in linearized pE3KN (Fig. 1A) in a competitive PCR. The RT and PCR conditions were as described in Materials and Methods except that the amounts of the RT product (cDNA) and the Ad competitor DNA included in the PCR were altered as described below. The reaction mixtures for lanes 2 to 9 contained a constant amount of cDNA (arrow b) which was a 10-fold dilution of the RT product obtained from the livers of LPS-treated transgenic animals. The competitor DNA was serially diluted by 10-fold decrements from lanes 2 to 8 (arrow a), starting with 300 pg in lane 2. Lane 9 contained no competitor DNA. The point of equivalence for amplified genomic and cDNA is between lanes 5 and 6, which represent 0.3 and 0.03 pg of the competitor DNA. Lanes 10 to 17 represent the cDNA diluted 10 times from the livers of transgenic animals infected with Ad7001 for 16 h with the amount of competitor DNA serially diluted 10-fold from 300 pg in lane 10. Lane 17 contained no competitor DNA. The equivalency point for the liver-specific and competitor DNA lies between lanes 10 and 11, which represent 300 and 30 pg of competitor DNA. Lane 1 contains the 100-bp molecular size markers.

RNase protection, which represents another method to obtain quantitative data on the E3 transcripts, was applied selectively to some of the organs and tissue cultures from the transgenic animals. The nature of the RNA probe RPA1 used for these experiments is shown in Fig. 1B and (after electrophoresis) Fig. 5A, lane 14); it is protected by a region of the mRNAs near the promoter (Fig. 5A, arrow a). This probe is also protected by a fragment from Ad7001; however, due to a mutation introduced into the virus at nucleotide 291, an 8-base mismatch appears when protecting RNA is synthesized from this region and results in two equimolar RNAs designated by arrows b and c (Fig. 5A, lanes 3 and 5 to 7). Thus, this approach allows the simultaneous quantitation of the transgenic E3 promoter RNA and the abortive transcript from the Ad7001 E3 promoter which is absent when LPS is used for the induction (Fig. 5A, lanes 10 and 12). The results of tissue culture of transgenic kidneys and lungs show clearly that there

TABLE 1. E3 promoter activity (basal or induced) measured by quantitative RT-PCR in various transgenic organs and cells

| Source of mRNA | Organ | Tissue culture | Activity measured | | | Amt of competitor DNA (pg)[a] | Fold enhancement |
|---|---|---|---|---|---|---|---|
| | | | Basal | LPS induced | Ad7001 induced | | |
| Lung | | + | + | | | 0.05 | |
| Lung | | + | | | + | 7.8 | 156 |
| Lung | + | | + | | | ND | |
| Lung | + | | | + | | 0.62 | ≥62 |
| Kidney | | + | + | | | 0.13 | |
| Kidney | | + | | | + | 195 | 1,500 |
| Kidney | + | | | + | | 0.62 | |
| Small intestine | + | | + | | | 2.5 | |
| Small intestine | + | | | + | | 1.5 | 0 |
| Small intestine | + | | | | + | 3.1 | 0 |
| Liver | + | | + | | | ND | |
| Liver | + | | | + | | 0.1 | ≥10 |
| Liver | + | | | | + | 31 | ≥3,100 |
| Testis | + | | + | | | 5 | |
| Spleen | + | | + | | | ND | |
| Spleen | + | | | + | | 0.02 | ≥2 |
| Spleen | + | | | | + | 1.25 | ≥125 |
| Thymus | + | | + | | | 1.56 | |

[a] ND, none detected. The level of detection was 0.01 pg and was used to calculate fold enhancement in organs in which basal expression was not detectable. All numbers were determined by quantitative PCRs using twofold dilutions of competitor DNA.

is much more E3 mRNA in kidney cultures than in lung cultures after infection with Ad7001 (Fig. 5A, lanes 3 and 5 to 7, band a). The amount of promoter induction in the kidneys and lungs in vivo after LPS treatment is smaller, which is consistent with the quantitative RT-PCR results; however, the signal can be detected as band a, and there are no smaller b and c bands (Fig. 5A, lanes 10 and 12) as in the Ad7001-infected cultures. There was no basal expression of the transgene in the kidneys (lane 8); however, with RT-PCR it was readily detectable. This probably reflects the higher sensitivity of RT-PCR. Since Ad7001 is not delivered efficiently to the lungs and kidneys after i.p. injection, there is no induction in these organs in vivo (lanes 9 and 11). As a control for the integrity of the RNA, the β-actin results are shown at the bottom of the figure. In order to prove the origins of bands a, b, and c in Fig. 5, HeLa cells were infected with either Ad2 or Ad7001 and the RNase protection studies were repeated with the RPA1 probe. The results demonstrated that only band a appeared after Ad2 infection; only bands b and c appeared in Ad7001-infected cells (data not shown).

Results of assays for basal and Ad7001- and LPS-induced E3 transgene expression for additional organs are shown in Fig. 5B. Since the RNase protection probe had the same specific activity as in the experiment shown in Fig. 5A, the results are comparable. Basal expression was readily detectable in the stomach (lane 1), small intestine (lane 3), and large intestine (lane 6) but not in the liver (lane 9). Although the basal expression in the stomach and large intestine is considerable, there was an increase in expression due to LPS in the stomach (lane 2) or large intestine (lane 7). The small changes after LPS treatment or Ad7001 infection in the small intestine were similar to the results of quantitative PCR (Table 1). However, in the liver Ad7001 was a strong inducer of transgene expression (lane 11). The RNA samples used for RNase protection assays and competitive PCRs were prepared from the same homogenized tissue, and the results of both of these assays were concordant.

**Demonstration of E3 specific mRNAs in kidney and lung cultures from transgenic animals.** After demonstrating the promoter activity in various organs and tissue culture cells from the transgenic animals, other PCR primers (Fig. 1B) were utilized to measure the individual mRNAs transcribed from the complex E3 transcription unit. It could be shown that the primers for the mRNAs for the 10.4K (primers 53 and 99) and 14.7K (primers 53 and 98) protein detected products of the RT-PCR in kidney and lung tissue cultures after E3 promoter induction by Ad7001 infection (Fig. 6). Thus, the mRNAs for the 14.7K (Fig. 6A) and 10.4K (Fig. 6B) protein were synthesized and correlated with E3 promoter activity. However, there was an interesting difference between kidney and lung cultures when the mRNAs for the gp19K and 11.6K protein were measured with primers 53 and 88 (Fig. 6C). These mRNAs can be measured with identical primer pairs, and the molar ratios of these two mRNAs will be reflected in the ratios of the amplified cDNA products, similar to what was possible when the levels of a single cDNA and a competitor DNA were compared as described above for the PCR quantification experiments. The results showed the predominance of the transgenic gp19K amplified cDNAs in the kidney, as anticipated from previous data that the gp19K mRNA and protein are the predominant products from the E3 region in cells infected with wt Ads in tissue culture (Fig. 6C). However, in the transgenic lung fibroblast culture there was a predominance of the 11.6K amplified cDNA products. This result indicates that organ-specific factors control the steady-state levels of the various E3 mRNAs. This experiment clearly shows that the ratios of mRNAs for the gp19K and 11.6K protein are different in transgenic kidney and lung cultures.

**Ad gp19K can be synthesized from the Ad E3 transgene.** The kidney and lung cultures from the transgenic animals were studied for the synthesis of gp19K both in the basal state and after activation of transcription by infection with Ad7001. As shown in Fig. 7, the synthesis of gp19K is readily apparent by immunoprecipitation of radioactively labeled proteins from kidney cultures (lane 1) after infection with Ad7001 but not from similarly processed lung cultures (lane 3), which may reflect the transcriptional results (promoter activity and/or selective splicing) described above. Although the transgenic lung culture was not stimulated to produce detectable amounts of gp19K by coinfection with Ad7001, the lung culture from

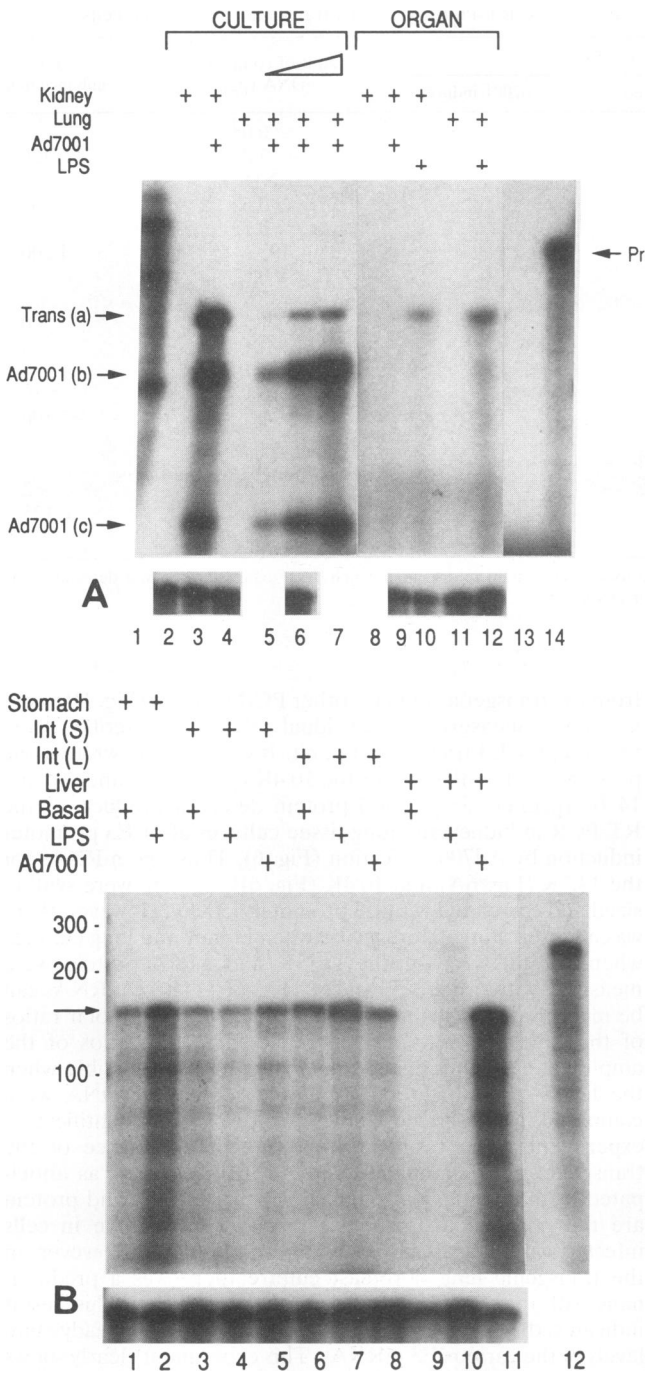



FIG. 5. RNase protection studies to quantify the amount of E3 transcripts. (A) The RPA1 RNA probe (Fig. 1B) labeled with [$^{32}$P]UTP (lane 14) was hybridized with RNA purified from either E3 transgenic tissue cultures or organs of transgenic mice as described in Materials and Methods to determine the amount of homologous sequences expressed either basally or after stimulation with LPS or Ad7001 infection. Lane 1, 100-bp molecular size markers; lanes 2 and 3, RNAs (3.5 μg) from kidney cultures either uninfected or infected with Ad7001 ($3 \times 10^{11}$ virions) for 16 h, respectively; lanes 4 to 7, RNAs from lung cultures (lane 4, uninfected [3.5 μg of RNA]; lanes 5 to 7, Ad7001 infected [1.4, 3.5, and 12.3 μg of RNA, respectively]); lanes 8 to 10, 7.5 μg of RNA from an E3 transgenic kidney (in vivo) (lane 8, from an untreated animal; lane 9, from a mouse infected with Ad7001 [$3 \times 10^{11}$ virions]; lane 10, from an animal injected with 400 μg of LPS i.p.); lanes 11 and 12, 7.5 μg of RNA from the lung of a transgenic animal untreated (lane 11) and treated with LPS (lane 12); lane 13, unprotected probe with RNase; lane 14, not treated with RNase. Arrow a designates the expected size of the protected Ad probe. The bands designated by arrows b and c are the two segments of the Ad7001 abortive transcript from the truncated DNA. The sequence at the first ATG site was mutated in Ad7001, which produced an 8-base mismatch with the RPA1 RNA. This mismatch was sufficient to serve as a substrate for cleavage by the RNase during the nuclease protection studies and yielded two equimolar bands upon electrophoresis. For RNase protection with the β-actin probe 2.5 μg of each RNA was used. The results are shown at the bottom of the panel, with lane 6 representing the results for the three dilutions of the same RNA shown in lanes 5 to 7. (B) The RPA1 probe with the same specific activity as described for panel A was used to quantify the amount of E3 transgene mRNA in the stomach, liver, small intestine, and large intestine. Conditions for basal and Ad7001- and LPS-stimulated expression were identical to those for panel A. Each assay was performed with 10 μg of RNA. Lanes 1 and 2, samples from the stomach (basal and LPS stimulated, respectively); lanes 3 to 5, small intestine (basal and LPS and Ad7001 stimulated, respectively); lanes 6 to 8, large intestine (basal and LPS and Ad7001 stimulated); lanes 9 to 11, liver (basal and LPS and Ad7001 stimulated); lane 12, undigested RPA1 probe. Five micrograms of RNA was used for RNase protection with the β-actin probes except for lanes 1 and 2, for which 10 μgs was used. The results are shown in the bottom panel.

normal animals was capable of making gp19K when infected with wild-type Ad2 (lane 5). Thus, the tissue-specific restriction that appears to decrease the amount of gp19K mRNA in transgenic lung (Fig. 6C) could be overcome in normal lung fibroblasts infected with wild-type virus at high multiplicities.

## DISCUSSION

The Ad2 E3 genes have been successfully introduced into transgenic animals, and expression of a variety of E3 mRNAs and gp19K has been detected. Widely distributed basal expression of transgenic mRNA was demonstrated in the thymus, stomach, small intestine, large intestine, kidneys, testes, and uterus. Expression in lymphoid organs such as the thymus was anticipated on the basis of the previously demonstrated activity of the E3 promoter in lymphoid cells (51) and induction in tissue culture by TNF-α (30), which is expressed constitutively in the thymus (18); however, there was no basal expression in another lymphoid organ, the spleen. The unanticipated observation that the E3 genes are strongly expressed at all levels of the gastrointestinal tract suggests that the E3 region is important for the initial replication at the port of entry of many Ads. These observations also point out the importance of studying promoter activation in vivo with intact viral genes even after information is available from in vitro constructs. The wide organ distribution of E3 expression is in marked contrast to the previously reported limited expression of the transgenic Ad E2 promoter driving a reporter gene, which was active only in some cells of the ovary (12).

The E3 promoter when delivered within Ad virions requires the viral E1A for efficient expression. We have utilized the Ad7001 (E1A+/E3−) E3 deletion to deliver the E1A for some of the transactivations because this virus is not cytotoxic in mouse cells, does not inhibit host protein synthesis, does not kill mice, and provided a distinct product for the RNase protection experiments. However, analysis of promoter-proximal sequences has indicated other mechanisms for activating the E3 promoter in the transgenic animals. We utilized LPS through its well-known stimulation of the production of TNF-α, which has been shown to activate the E3 promoter in transfected constructs, probably through the NF-κB mecha-

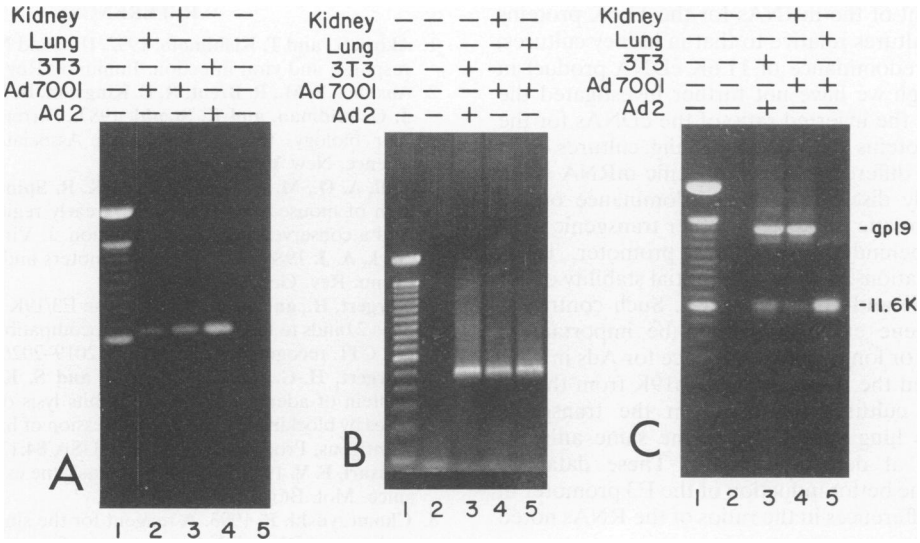



FIG. 6. Demonstration of the variety of Ad E3-specific mRNAs. The patterns of expression of various mRNAs from the Ad E3 expressed in tissue culture of transgenic kidney and lung fibroblasts were demonstrated with selected PCR primers. The E3 promoter was stimulated by coinfection with Ad7001 (3,000 virions per cell) ($E1A^+$ $E3^-$). These patterns were compared with those of infections of NIH 3T3 cells with Ad2 ($E1A^+$ $E3^+$) (A) Results with primer pairs 53 and 98 (Fig. 1B) to detect the 14.7K spliced mRNA by RT-PCR as described in Materials and Methods using undiluted cDNA and 46 cycles for PCR. Lane 1, 100-bp markers; lanes 2 to 4, cDNA from lung, kidney, or NIH 3T3 cells at 16 h after infection with the designated virus; lane 5, reaction mixture as a negative control. The expected band of 621 bp was demonstrated in the three positive lanes. (B) Primers 53 and 99 were used to detect the 10.4K mRNA. The expected size of the PCR product was 436 bp. Lane 1, markers; lane 2, negative control for the reaction; lanes 3 to 5, components as marked and described for panel A. (C) RT-PCR products detected when primers 53 and 88 were utilized to detect both the gp19K (1,390-bp) and 11.6K (601-bp) cDNAs as designated; these reactions were otherwise identical to those in panel A.

nism (18). TNF-α presumably acts in vivo in the same manner; however, it is unclear whether it is made in each organ that is stimulated by systemic LPS or the cytokine is extracted from the circulation. A local effect might explain the good induction in the liver, where the Kupfer cells, equivalent to fixed macrophages elsewhere in the body, might provide ample TNF-α for activating hepatocytes. It is also possible that the Kupfer cells themselves are providing all the E3 activity measured in the liver. We have not yet completed in situ experiments to answer this question. It is also possible that the LPS has an effect through the NF-IL6 mechanism whereby the IL-6 cytokine could activate NF-IL6, which has a cognate binding site in the E3 enhancer. NF-IL6 is inducible by LPS in many organs (1).

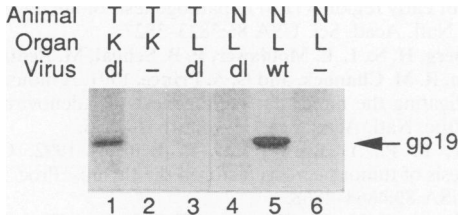



FIG. 7. Expression of Ad gp19K in transgenic tissue. Short-term tissue cultures were produced from transgenic (T) kidney (K) and lung (L) or from normal lung (N). These cells were infected either with Ad7001 (dl) or with Ad2 (wt) at 50 PFU per cell and labeled with [$^{35}$S]methionine for 5 h at 16 h postinfection, and lysates were made as described in Materials and Methods. Transgenic kidney cultures infected with Ad7001 produced a radioactive band that was specifically immunoprecipitated with rabbit polyclonal antibody prepared against Ad2 gp19K (lane 1); however, there was no basal expression of gp19K in transgenic kidney cultures without induction (lane 2). In contrast, lung cultures obtained from transgenic mice and infected with the Ad7001 (lane 3) did not produce detectable amounts of gp19K. Lane 4, negative control from normal mouse lung infected with Ad7001; lane 5, gp19K produced as a marker in Ad2-infected normal lung fibroblast cultures; lane 6, the same cells uninfected.

Among the organs tested, LPS induction was the strongest in the kidneys and lungs. It is interesting to note that the TNF promoter in transgenic animals is also induced in the kidneys and lungs at the highest levels (18). Quantitative comparison of induction by LPS and E1A (delivered in Ad7001) showed a much higher induction by E1A in organs such as the liver (≥3,100-fold) and a 1,500-fold increase in isolated tissue cultures of primary kidney cells. This is much higher than the 30- to 40-fold activation of the promoter in secondary lines such as HeLa (4). In addition, there was a marked difference of the level of E1A induction of the E3 promoter between kidney (1,500-fold) and lung (150-fold) cultures. However, in the lungs in vivo, there was no induction by Ad7001 but there was induction by LPS. This probably reflects the relative distribution of virus in the animal following i.p. infection of a poorly replicating virus, rather than an intrinsic property of the promoter or its activators. The in vivo induction by LPS in these experiments was achieved in the absence of E1A, which was present in the tissue culture induction of the E3 promoter by TNF-α previously reported (30). Thus, it appears that TNF-α can affect the E3 promoter without the help of any other viral genes.

The differential pattern of transcript expression in lung tissue culture wherein the 11.6K protein mRNA appears to be made in larger amounts than the major transcript gp19K mRNA cannot be explained by promoter activity, since they are alternatively spliced products of the same initial RNA. We have assumed that either the overall level of gp19K mRNAs is

reduced or the amount of the mRNAs for the 11.6K proteins is increased in lung cultures relative to that in kidney cultures, on the basis of the predominance of 11.6K cDNA product in the RT-PCR. Although we have not further investigated the mechanism to explain the inverted ratio of the cDNAs for the gp19K and 11.6K proteins in transgenic lung cultures, it is possible that there is differential tissue-specific mRNA splicing. We have recently discovered the predominance of the 11.6K mRNA in pancreatic islet cells in other transgenic mice with the E3 region behind the rat insulin promoter. There might be other explanations, such as differential stability of the mRNAs for the 11.6K and gp19K proteins. Such control of organ-specific viral gene expression might be important in either acute virulence or long-term persistence for Ads in vivo. We have demonstrated the production of gp19K from the E3 transgene in kidney cultures derived from the transgenic animals. However, in lung cultures from the same animals, gp19 was not made at detectable levels. These data are consistent with both the better induction of the E3 promoter in the kidneys and the differences in the ratios of the RNAs noted in lung cultures.

One of the goals in constructing the transgenic animals was to study the effects of Ad E3 in a natural Ad infection in the mouse. Since the human Ads infect but do not replicate well in murine tissues and the mouse Ad MAV-1 lacks an E3 region with the ORFs present in the human Ads (40), the transgenic animals containing the human Ad2 E3 ORFs will substitute for the genes missing in the MAV-1 or MAV-2 infections. Our data show that E1A from MAV-1 can transactivate the transgenic E3 promoter in many organs, including those that are the sites of MAV-1 pathology (data not shown). In addition, the E3 promoter is inducible in liver, which is the site of the Reye syndrome pathology that we have previously demonstrated after MAV-1 infection in SCID animals (36). Constitutive E3 expression in the intestine should be very useful for studies of MAV-2 pathogenesis, since this virus is an intestinal pathogen, as well as of MAV-1, because the intestine is probably the portal of entry (35). The effects of the E3 genes can now be measured both in suckling animals that undergo acute and fatal disease and in adult mice that have less acute disease but develop persistent infection, especially of the urinary tract.

It is also possible that the transgenic mice described in this report will be useful to probe the roles of class I MHC and TNF-α in the pathogenesis of other infectious agents as well as to study diseases in which LPS has an effect on survival or organ pathology. From animals in which the TNF-α 55K receptor has been deleted by a gene knockout technique (43), it appears that intracellular parasites like *Listeria monocytogenes* are more virulent, but the same animals are protected from the adverse effects of endotoxin produced by gram-negative bacteria or the superantigen staphylococcus enterotoxin B. Since the effects of TNF-α are protean and work through several intracellular pathways, it may be possible to dissect some of these through the use of the transgenic animals reported in this work.

## ACKNOWLEDGMENTS

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